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14. ABSTRACT

The long-term objective of this research is to develop a device for treating spinal cord injury. The specific aims of the proposed study are to test new types of collagen biomaterials. Moreover we will be investigating the effects of incorporating genes from nerve growth factors into the collagen scaffolds and seeding the scaffolds with neurogenic cells. The standardized defect site is a 5-mm gap in the rat thoracic spinal cord. Our principal method of evaluation is histomorphometry. During the past project year the following were accomplished, toward achieving the objectives of determining the effects of selected design variables on the reparative processes in spinal cord defects: 1) investigation of nanoparticles, as carriers of genes for neurotrophic factors, to be incorporated into collagen scaffolds for implantation into spinal cord defects; 2) development of an injectable collagen formulation as a carrier for plasmid DNA encoding for neurotrophic factors; 3) formulation of collagen scaffolds containing hyaluronic acid; and 4) investigation of the conditions *in vitro* for the neuro-differentiation of neural stem cells.

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I. INTRODUCTION

The long-term objective of this research is to develop a device for treating spinal cord injury. The specific aims of the proposed study are to test new types of collagen tubes and porous collagen scaffolds. Moreover we will be investigating the effects of incorporating genes from nerve growth factors into the collagen scaffolds and seeding the scaffolds with marrow-derived mesenchymal stem cells. The standardized defect site is a 5-mm gap in the rat thoracic spinal cord. Our principal method of evaluation is histomorphometry.

Our supposition is that an appropriate synthetic substrate (*i.e.*, the collagen scaffold) will mimic or perhaps improve upon the documented ability of peripheral nerve grafts to promote the regrowth of injured spinal axons, and that the seeded stem cells will differentiate under the influence of the endogenous regulators to a support cell phenotype. Collagen-based materials are the bio-inspired biomaterials being used for the fabrication of the tube, covering film and porous scaffold.

II. BODY

During the past project year, research focused on the following:

1) investigation of nanoparticles, as carriers of genes for neurotrophic factors, to be incorporated into collagen scaffolds for implantation into spinal cord defects;
2) development of an injectable collagen formulation as a carrier for plasmid DNA encoding for neurotrophic factors;
3) formulation of collagen scaffolds containing hyaluronic acid; and
4) investigation of the conditions *in vitro* for the neuro-differentiation of neural stem cells.

A. Gelatin Nanoparticles as Carriers of Genes for Neurotrophic Factors

1. Background

Our prior work demonstrated that the collagen scaffold to be employed as an implant in spinal cord defects could serve as a delivery vehicle for genes encoding for neurotrophic factors. To further advance that work, we investigated the incorporation of plasmid DNA encoding for neurotrophic factors, insulin-like growth factor (IGF)-1 and osteogenic protein (OP)-1, into gelatin and chitosan nanoparticles which could be used to supplement the scaffold. This approach has the potential to enhance non-viral gene transfer and to readily deliver genes for several growth factors.

2. Materials and Methods

Gelatin was chemically modified by grafting amino groups to carboxyl groups using a carbodiimide chemical treatment. The plasmids encoding for IGF-1 and enhanced green fluorescent protein (pEGFP) were amplified in *Escherichia coli* host strain DH5α, and purified by column chromatography. The plasmid for EGFP was used as a reporter gene to visualize the transfection of chondrocytes by fluorescence microscopy. The size of the pEGFP was 4.7 Kb and the pIGF-1 was between 6 and 7 Kb in size. The yield, purity, and integrity of the prepared plasmids were evaluated with an ultraviolet spectrophotometer and by gel electrophoresis.

Cationized gelatin-plasmid IGF-1 nanoparticles (CGPIN) were prepared by complex coacervation, which involves separation by the interaction of two oppositely charged colloids. Nanoparticles, which were prepared with either the pIGF-1 or the pEGFP, were used without further purification. The nanoparticles containing pEGFP were prepared with cationized gelatin using a gelatin:plasmid weight ratio of 250:1, whereas a variety of ratios were investigated when

making nanoparticles encapsulating pIGF-1. As a control group, nanoparticles were also synthesized using the original (non-cationized) gelatin material.

Environmental scanning electron microscopy was used to investigate the size and shape of the nanoparticles. The particle size distribution was determined by a dynamic light scattering technique. The zeta potential of the nanoparticles, with different weight ratios of gelatin to plasmid was also measured.

For transfection of cells (chondrocytes) with gelatin nanoparticles incorporating either plasmid, the medium was removed and replaced with a 250 μl suspension of nanoparticles diluted in a serum-free medium. Five hours later, the nanoparticle solution was removed and replaced with fresh serum-free medium that did not contain nanoparticles. Based on an average nanoparticle diameter and a gelatin density of about 1 g/ml, we estimated that the number of nanoparticles added to the monolayer chondrocytes was on the order of 10^{12}. Assuming that the cultures to which the nanoparticles were added contained approximately 100,000 cells, the number of nanoparticles per cell was estimated to be 10^{7}.

Monolayers that were treated with cationized gelatin nanoparticles incorporating pEGFP at a weight ratio of 250:1 and a plasmid amount of 10 μg per well were examined by transmitted fluorescence microscopy 48 hours after transfection in order to visualize successful gene transfer to cells using these nanoparticles.

For IGF-1 transfected monolayers, the effects of cationized gelatin:IGF-1 ratio and plasmid amount added to each well were investigated. Five different cationized gelatin:IGF-1 ratios were investigated (by weight): 150:1, 200:1, 250:1, 300:1, and 400:1. For these groups, a constant plasmid load of 10 μg per well was used. In experiments in which plasmid amount was the main variable, the five different plasmid amounts used were 2, 5, 8, 10, and 12 μg of plasmid per well, at a constant cationized gelatin to pIGF-1 weight ratio of 250:1. Control conditions consisted of pIGF-1 only (10 μg) or no treatment (just added serum free medium). Nanoparticles synthesized using unmodified (original) gelatin at a gelatin:IGF-1 ratio of 250:1 was also included as an experimental condition to determine the effect of cationization on nanoparticle characteristics and transfectibility. The serum-free medium from IGF-1 transfected cultures was collected at 144 hrs after transfection and assessed for the presence of IGF-1 protein (n = 4) with a human IGF-1 sandwich ELISA kit.

3. Results and Discussion

ESEM (Fig. 1) revealed different sizes and morphologies of the pIGF-1-containing nanoparticles prepared with cationized gelatin versus regular gelatin (using the same gelatin:plasmid weight ratio of 250:1). The cationized gelatin nanoparticles (Fig 1a-b) generally appeared to be of spherical shape. The small size of the cationized gelatin nanoparticles precluded definitive measurement of their diameter by ESEM. The particles generally appeared, however, to be less than 200 nm, which was consistent with the measurements made by dynamic light scattering. In contrast, the original gelatin formed micro-scale particles with spherical and ellipsoid shapes (Fig 1c-d), with the average diameter (and long axis) appearing to be from 10 μm to greater than 20 μm. For both the cationized and non-cationized gelatin:plasmid preparations, the nanoparticles and microparticles, respectively, generally appeared to be of uniform size.

Dynamic light scattering revealed that the size of the cationized gelatin nanoparticles ranged from 7 nm to 387 nm, with an average diameter of 172 nm. The original gelatin particles displayed a wider size range extending to greater than 1.8 μm, larger than could be evaluated by dynamic light scattering.

The zeta potential of the naked plasmid IGF-1 solution was -48 ± 2 mV (mean ± standard error for 5 runs of the same sample). When the plasmid was coupled with different amounts of the positively charged cationized gelatin (+18 ± 0.7 mV for cationized gelatin alone), the zeta potential went from a negative value to a positive value and increased by approximately 50-58 mV. Interestingly, with the increasing cationized gelatin:plasmid weight ratio, the surface charge of the nanoparticles did not show a significant change. Cationized gelatin pIGF-1 nanoparticles displayed a 4-fold higher positive charge compared to particles made with the unmodified gelatin (GPIN) using a 250:1 gelatin to plasmid weight ratio.

There was no noticeable fluorescence from the cells treated with the nanoparticles prepared with the original (non-cationized) gelatin particles containing the pEGFP. In contrast numerous cells in the group transfected with the cationized gelatin-pEGFP nanoparticles were found to fluoresce indicating successful transfection and gene expression.

There was a clear effect of varying the weight ratio of gelatin to pIGF-1 on gene transfer and subsequent IGF-1 release in the medium (Fig. 2a). Optimal IGF-1 expression was recorded for gelatin to plasmid weight ratios of 200-300:1. There was a 5-fold elevation in the amount of IGF-1 produced from the group treated with nanoparticles synthesized at a weight ratio of 250:1 compared to the control group that was treated with pIGF-1 alone (Fig. 2a). One-factor ANOVA revealed a significant effect of the weight ratio of cationized gelatin to pIGF-1 on the amount of IGF-1 synthesized by the cells ($p < 0.0002$; power = 1). Fisher's PLSD post-hoc testing demonstrated that all plasmid ratios had statistically significant elevations of IGF-1 production over the control condition treated with plasmid only (i.e. no incorporation in nanoparticles, $p < 0.04$). Among the gelatin:plasmid weight ratios, there was no statistically significant difference between the 200:1, 250:1, and 300:1 groups, but there was a significant difference comparing these groups with the 150:1 group ($p < 0.03$), and comparing the 250:1 and 300:1 groups versus the 400:1 group ($p < 0.02$).

There was also a notable effect of varying the amount of plasmid added to each well on IGF-1 produced by the transfected chondrocytes (Fig. 2b). There was a gradual increase in IGF-1 expression with increasing plasmid load. Linear regression analysis demonstrated a correlation between IGF-1 expression and plasmid load ($R^2 = 0.65$). One-factor ANOVA showed a significant effect of plasmid amount added per well on IGF-1 expression ($p < 0.0001$; power = 1). Post-hoc testing revealed that there was a statistically significant elevation in IGF-1 produced for all plasmid loads except the 2 μg load when compared to the control group. The 12 μg load showed a statistically significant elevation of IGF-1 expression above the plasmid load groups of 8 μg or less ($p < 0.02$). The 10 μg load showed a significant elevation above plasmid loads of 5 μg or less ($p < 0.01$). There was, however, no significant difference between the 5 and 8 μg plasmid load groups or the 10 and 12 μg groups.

The difference between using unmodified (non-cationized) gelatin and cationized gelatin nanoparticles for the transfection of the chondrocytes was clearly demonstrated (Fig. 2c). There was a 5-fold elevation in the amount of IGF-1 produced by transfected cells when using the cationized gelatin nanoparticles (Fig. 2c). ANOVA showed a statistically significant difference in the IGF-1 expression between the groups ($p < 0.003$; power = 0.99).

The results of the present study demonstrated the benefits of altering the charge of gelatin through cationization, with respect to its use as a delivery vehicle for plasmid IGF-1 for non-viral gene transfer. When complexed with the plasmid, unmodified gelatin tended to form particles of substantially larger size and broader size range. Increasing the positive charge of gelatin may

have enabled it to condense the pIGF-1 such that smaller (nanometer-sized) particles could be produced.

Of interest were the findings demonstrating the difference in the functionality of the cationized and non-cationized gelatin particles as transfection agents for the plasmids encoding EGFP and IGF-1. Virtually no fluorescence was detected in chondrocytes treated with the marker gene, EGFP, incorporated in the unmodified gelatin particles, while many cells were transfected with the pEGFP using the cationized gelatin nanoparticles. This difference was also demonstrated by the 5-fold difference in expression of IGF-1 between groups treated with the cationized gelatin-pIGF-1 versus the non-cationized-pIGF-1 particles indicating that there are important functional differences imparted by charge modification, perhaps owing to a difference in particle size or surface charge. A higher positive surface charge may increase interactions with the negatively charged cellular membrane and a smaller particle diameter may increase probable entry into to cell, resulting in enhanced gene expression. Additional studies will be required to provide a deeper understanding of the transfection mechanisms that are responsible for the differences in the behavior of the cationized and non-cationized particles.

The present findings also revealed an optimal cationized gelatin:pIGF-1 weight ratio range for transfecting adult articular chondrocytes, with maximum IGF-1 expression recorded for weight ratios of 200-300:1. The surface charge of the nanoparticles made with the varying weight ratios, however, may not have been a significant factor influencing gene transfer to chondrocytes as the surface charge did not seem to change significantly using different weight ratios. It will be useful in future work to evaluate other potential mechanisms related to the ability of these nanoparticles to transfect cells, such as particle size and morphology and the kinetics of plasmid release from nanoparticles prepared with other gelatin:plasmid weight ratios.

The current investigation demonstrated a nearly linear increase in IGF-1 production by cells with increasing plasmid load applied to the cultures when using cationized gelatin nanoparticles. Based on an estimated cell number in each well of about 100,000 cells and using a cationized gelatin:IGF-1 weight ratio of 250:1, one would need about 50 pg of incorporated plasmid within cationized gelatin nanoparticles per cell for meaningful gene expression. The approximately 1 ng of IGF-1 collected in the first 144 hours after transfection (using a 250:1 cationized gelatin:pIGF-1 and a 10 μg plasmid load) is well below the minimum therapeutic levels generally found *in vitro* to elicit a response from chondrocytes using the recombinant IGF-1 as a medium supplement. However, this 1 ng level was achieved after only 5 hours of incubation of the cells with the nanoparticles. Moreover, in a contained defect *in vivo,* small levels of overexpressed IGF-1 concentrated locally may still be able to achieve therapeutic results.

In other related experimental work, we found that cells transfected with pIGF using cationized gelatin nanoparticles were able to maintain steady IGF-1 overexpression when subsequently seeded within collagen scaffolds for up to two weeks in 3-D culture.

4. Summary

The results of the present work demonstrate that gelatin nanoparticles can be synthesized to incorporate plasmid containing the IGF-1 gene, and can successfully transfect expanded cells in monolayer culture. Chemical modification of gelatin by cationization, varying the cationized gelatin to plasmid weight ratio, and varying the amount of plasmid added to the cells all significantly affect resulting gene expression and growth factor release kinetics.

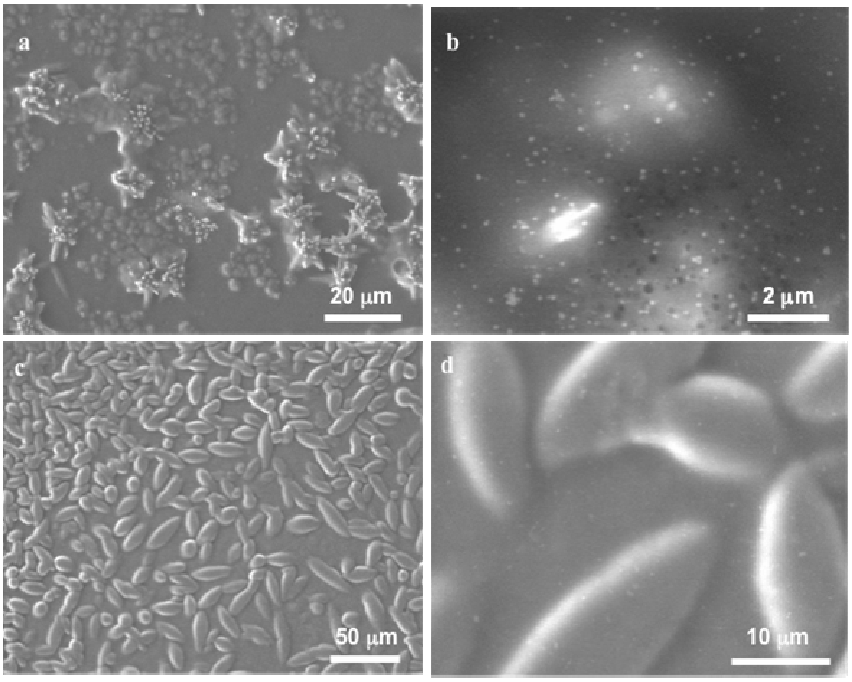


Fig. 1 (Top). Environmental scanning electron microcopy images of cationized gelatin-pIGF-1 nanoparticles (a, b) and non-cationized (original) gelatin-pIGF-1 microparticles (c, d), using a gelatin-plasmid weight ratio of 250:1.

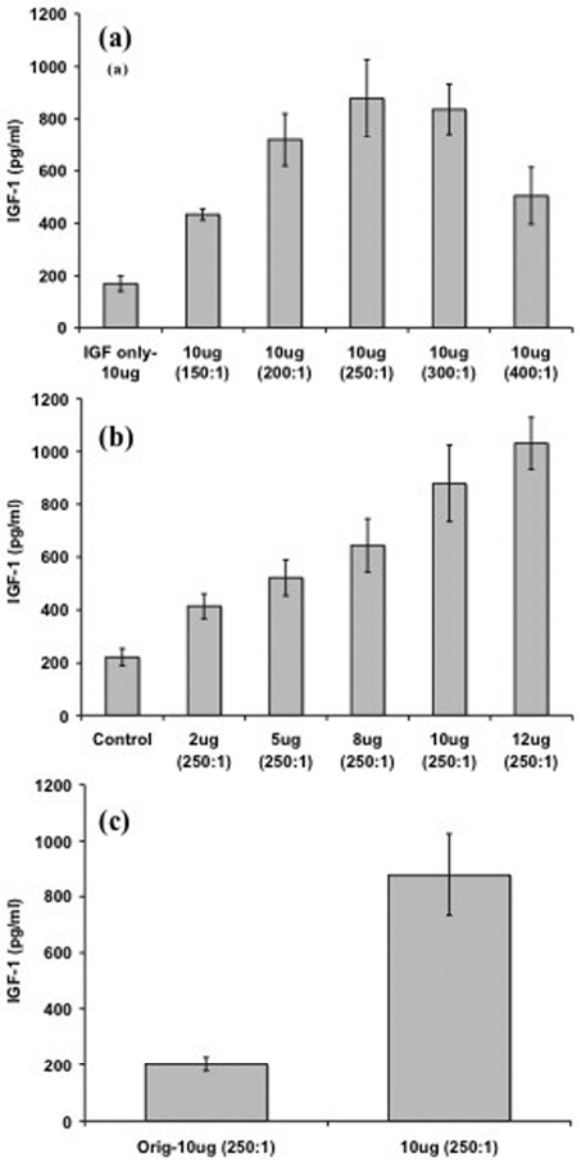


Fig. 2 (Right). IGF-1 protein released into the medium from monolayer chondrocytes 144 hours after treatment with gelatin-plasmid IGF-1 nanoparticles: varying the cationized gelatin to pIGF-1 weight ratio using a 10mg plasmid load (a); varying plasmid load using a 250:1 weight ratio (b); and comparing transfection using original gelatin particles vs. cationized gelatin nanoparticles (c). n=3-4; mean ± SEM.

B. Chitosan Nanoparticles as Carriers of Large Neurotrophic Growth Factor Plasmids

The objective of the study was to prepare chitosan nanoparticles incorporating the relatively large plasmid encoding for OP-1. The positive charge of chitosan acted to condense the relatively large negatively-charged OP-1 plasmid such that it could be incorporated into nanoparticles.

Incorporation of the plasmid into the chitosan nanoparticles did not affect the structural integrity of the plasmid as demonstrated by gel electrophoresis. The morphology and size of the nanoparticles were found to vary with the chitosan:plasmid weight ratio. Nanoparticles formulated with a chitosan:plasmid ratio of 10:1 were of uniformly small size (less than 250 nm) and spherical shape. These nanoparticles had a positive charge of about 20 mV.

FITC-labeled chitosan nanoparticles were found in virtually all of the cells (chondrocytes) after 24 hours of incubation with the nanoparticles, and confocal microscopy revealed FITC-related fluorescence in the nucleus of the cells. Although transfection of the cells was demonstrated by the fluorescence of cells treated with chitosan nanoparticles containing the

plasmid for the enhanced green fluorescence protein, cells transfected with nanoparticles incorporating the larger OP-1 plasmid did not show OP-1 expression measured by ELISA for up to 2 weeks in culture. These results indicate that although a large plasmid can be successfully incorporated within chitosan nanoparticles, the size of the plasmid incorporated within the nanoparticles may still significantly affect gene transfer to cells.

C. Injectable Collagen Particles as Carriers for Neurotrophic Gene Delivery

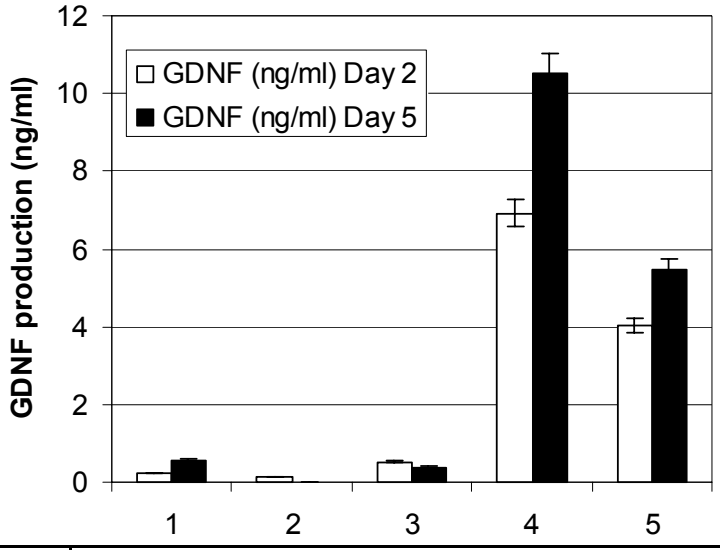
An injectable form of a type I collagen scaffold was prepared as a carrier for the neurogenic cells. The advantage of using a particulate, fibrous scaffold in an injectable form is that the large surface area of the particles allows for the delivery of a high density of attached cells. Once in the defect the cells will bridge the fibrous particles and synthesize matrix to produce a contiguous mass. The advantage of a fibrous scaffold over the many injectable polymers which gel *in situ* is that the latter immobilize the cells within a thin fibrillar (gel) network of nanometer-size pores, preventing their migration and proliferation, and preventing establishment of cellular communication with the cells in the surrounding host tissue. Advantages of the injectable scaffold, compared to injection of a cell suspension, include: 1) retention of cells, attached to the scaffold particles, in the lesion; 2) *in vivo* formation of a contiguous framework facilitating infiltration of host cells; and 3) the collagen particles can serve as carriers of plasmid DNA encoding for neurotrophic factors for the non-viral transfection of AMP cells and of host cells.

The basic collagen scaffold was prepared from porcine type I/III collagen powder (Geistlich Biomaterials, Wolhusen, Switzerland). A membrane form of the porcine type I/III collagen biomaterial is approved by FDA for treatment of periodontal defects, and is also approved in the European Union for the treatment several orthopedic problems. Particles of the collagen powder of selected size range, obtained by sieving, were sterilized and cross-linked by dehydrothermal treatment.

The collagen particles were suspended in the glial cell line-derived neurotrophic factor (GDNF) complexed with a lipid transfection reagent (GP2), and then removed and dried, prior to being added to the monolayer cultures of rat marrow-derived mesenchymal stem cells (MSCs); Figs. 3 and 4. The residual lipoplex, which was not taken up by the collagen particles, was also added to other MSC cultures. We found a small amount of expression GDNF by the MSCs (Fig. 3; Condition 1). Administration of the naked plasmid had no effect (Condition 2). There was a small increase in the expression of GDNF by the cells receiving the collagen particles containing the naked plasmid (Fig. 3; Condition 3), but a substantial increase in GDNF release to therapeutic levels after only 2 days in the cultures in which the collagen particles were impregnated with the GDNF-GP2 lipoplex (Fig. 3; Condition 5). GDNF over-expression was also seen in the cultures to which the residual lipoplex was added (Condition 4). These results demonstrate that an off-the-shelf injectable collagen particle, containing GDNF plasmid, can be prepared as a carrier for neurogenic cells.

D. Fabrication and Characterization of Porous Hyaluronic Acid-Collagen Composite Scaffolds

Hyaluronic acid (HA) plays a vital role in neural tissues and has been shown to have positive biological effects on cell behavior *in vitro*. To begin to determine if these benefits can be accessed if HA is incorporated into our collagen-based scaffolds for tissue engineering, HA-collagen composite matrices were prepared, and selected properties evaluated.



#	Conditions
1	MSCs alone
2	MSCs + pGDNF
3	MSCs + pGDNF + collagen particles
4	MSCs + pGDNF-GP2 (remaining GP2 lipolex)
5	MSCs + pGDNF-GP2 + collagen particles

Fig. 3 GDNF in the medium of monolayer cultures of MSCs transfected under various conditions; n=6.

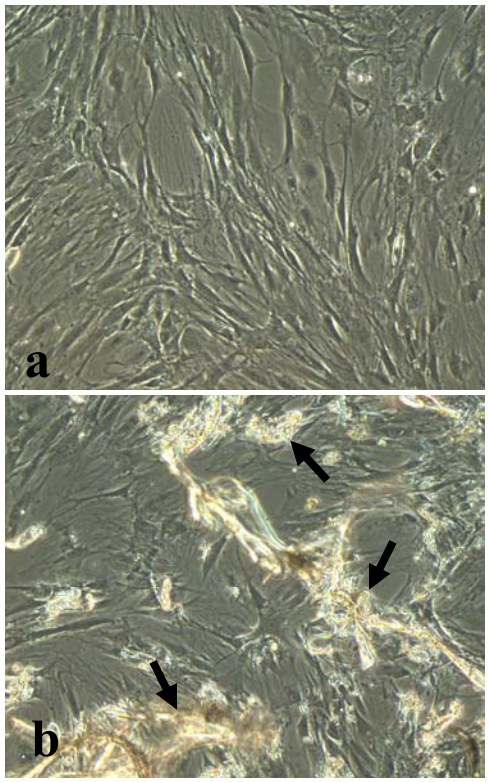


Fig. 4 Light micrographs of adult rat MSCs in monolayer after 2 days (a) in Condition 1 in Fig. 3, and (b) in Condition 5; arrows collagen particle.

HA-collagen scaffolds were cross-linked with carbodiimide and loss rates of HA in culture medium assessed. Scaffold pore structures were evaluated by light and electron microscopy.

Homogenous HA-collagen slurries were achieved when polyionic complexes were suppressed. HA was uniformly distributed through the scaffolds, which demonstrated honeycomb-like pores with interconnectivity among pores increasing as HA content increased. Virtually all of the HA added to the collagen slurry was incorporated into the composite scaffolds that underwent a 7-day cross-linking protocol. After 5 days in culture medium, the HA content in the scaffolds was 5-7% regardless of initial HA loading.

This study contributes to the understanding of the effects of HA content, pH, and cross-link treatment on pore characteristics and degradation behavior essential for the design of HA-collagen scaffolds.

E. Neurogenic Differentiation of Neural Stem Cells Growing in a Collagen Scaffold

One of our hypotheses is that certain biomaterial scaffolds can serve as regulators of cell function including the differentiation of stem cells. In order to begin test this hypothesis we compared the differentiation of fetal rat neural stem cells (NSCs) into neuronal cells in monolayer and in collagen sponge-like scaffolds (Fig. 5). The NSCs were expanded in number in monolayer in expansion medium for 4 days and then seeded into a type I/III collagen sponge-like scaffold. The cell-seeded constructs were cultured in expansion medium for another 2 days in order to allow time for cell attachment before being cultured in a neurogenic differentiation

medium for 6 days. The results (Fig. 5) demonstrated that the NSCs differentiated into neuronal cells in the 3-dimensional environment. Work in progress is investigating the effects of the chemical composition of the scaffold on the differentiation process.

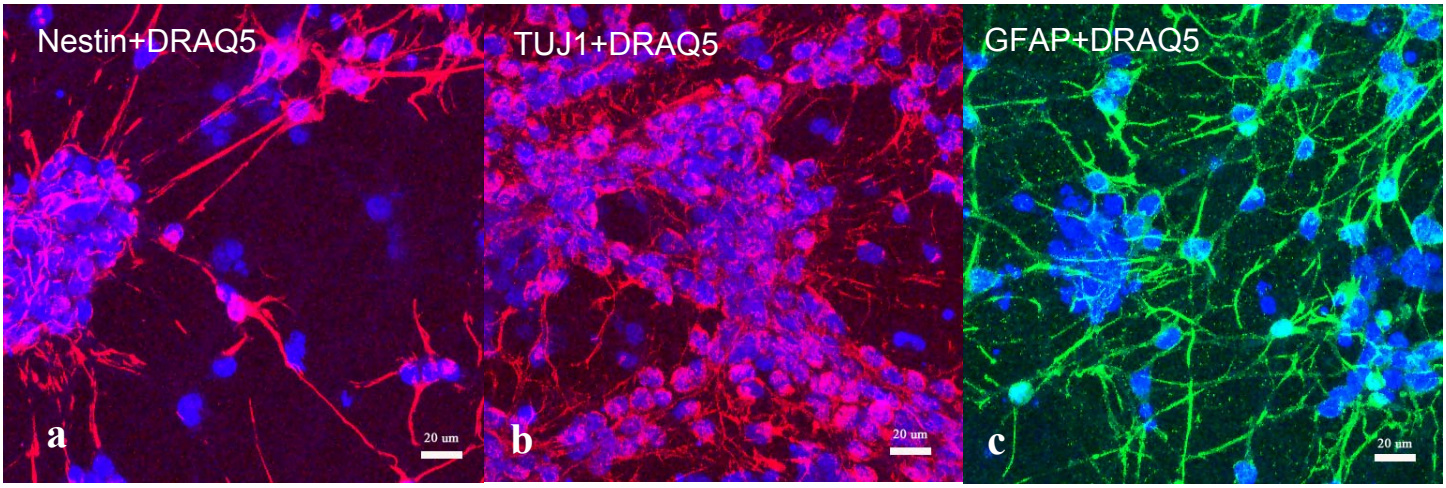


Fig. 5. Confocal images of rat embryonic neural stem cells which have undergone neuronal cell differentiation while growing in a type I/III collagen scaffold in a neurogenic medium. a) Nestin (red) is a marker for neural progenitor cells. b) TUJ1 (red) is a cytoskeletal protein found in immature neurons. C) GFAP (green) is a marker for astrocytes. GRAQ5 (blue) is a stain for nucleii.

III. KEY RESEARCH ACCOMPLISHMENTS

- Gelatin and chitosan nanoparticles were demonstrated to be effective carriers of plasmid DNA encoding for neurotrophic factors for non-viral gene transfer.
- An injectable collagen scaffold was shown to be capable of delivering the gene for a neurotrophic factor to mesenchymal stem cells.
- Methods were determined for the neuro-differentiation of neural stem cells grown in collagen scaffolds *in vitro*.

IV. REPORTABLE OUTCOMES

Tang S and Spector M. Incorporation of hyaluronic acid into collagen scaffolds for the control of chondrocyte-mediated contraction and chondrogenesis. Biomed. Mater. 2007;2:S135-S141.

Xu X, Capito RM, and Spector M. ' Delivery of plasmid IGF-1 to chondrocytes via cationized gelatin nanoparticles. J. Biomed. Mater. Res. 2008;84A:73-83.

Xu X, Capito RM, and Spector M. ' Plasmid size determines chitosan nanoparticle mediated gene transfer to chondrocytes. J. Biomed. Mater. Res. (In press).

Madaghiele M, Sannino A, Yannas I.V. and Spector M. Collagen-based matrices with axially oriented pores. J. Biomed. Mater. Res. (In press).

V. CONCLUSIONS

These results demonstrated the potential utility of nanoparticles, injectable collagen, and collagen scaffolds for the formulation of implants to treat defects in the spinal cord.

VI. REFERENCES

None

VII. APPENDICES

None